



Figure 1A

SEQ ID NO:1

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Figure 1B

SEQ ID NO:2

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Figure 1F

SEQ ID NO:6
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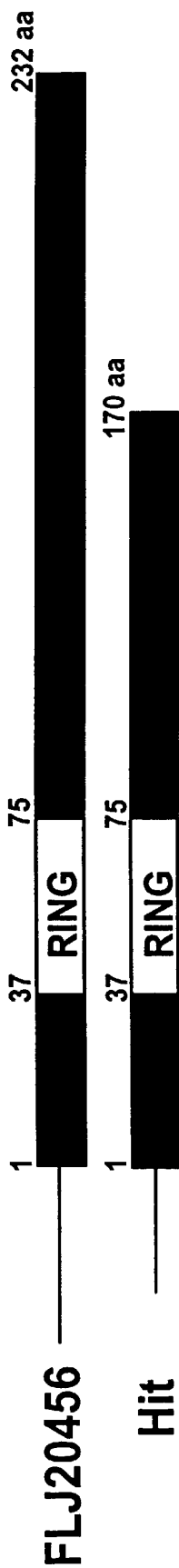
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SEQ ID NO:7
Mouse TRAC1 protein (3rd frame)

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FIGURE 2A

FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells



Original clone

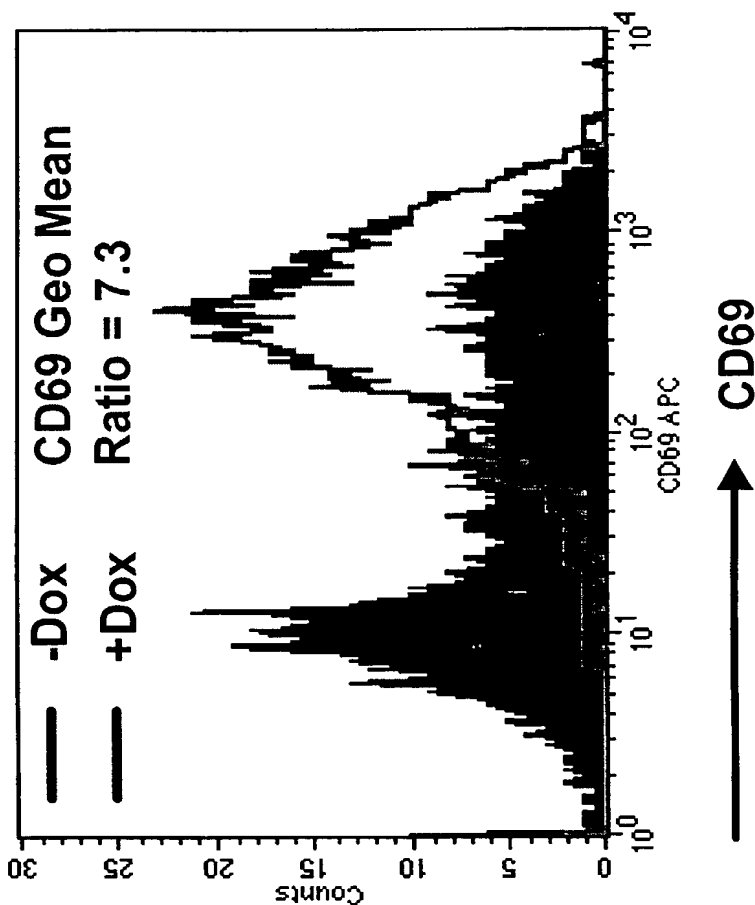


FIGURE 2B

**FLJ20456 Hit Inhibited anti-TCR Induced
CD69 Expression in Jurkat Cells**

Phenotype Transfer

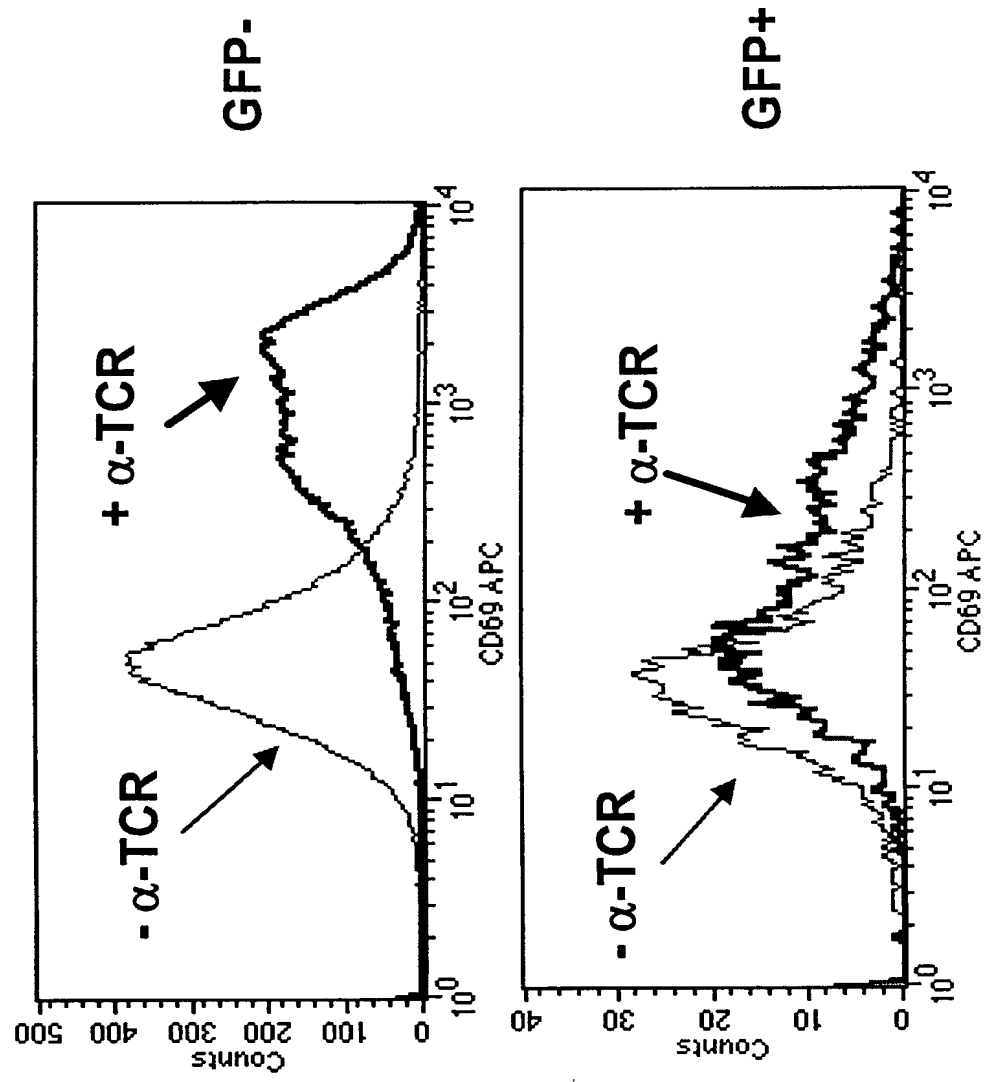


Figure 3A

232 aa

- # JurkatN 32H

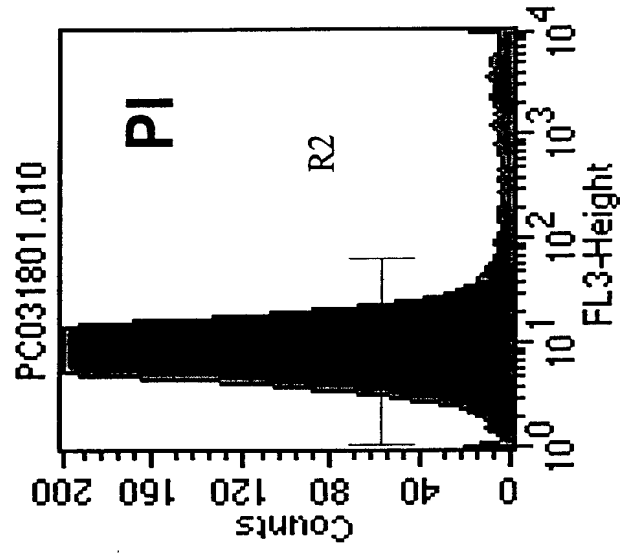


Figure 3B

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

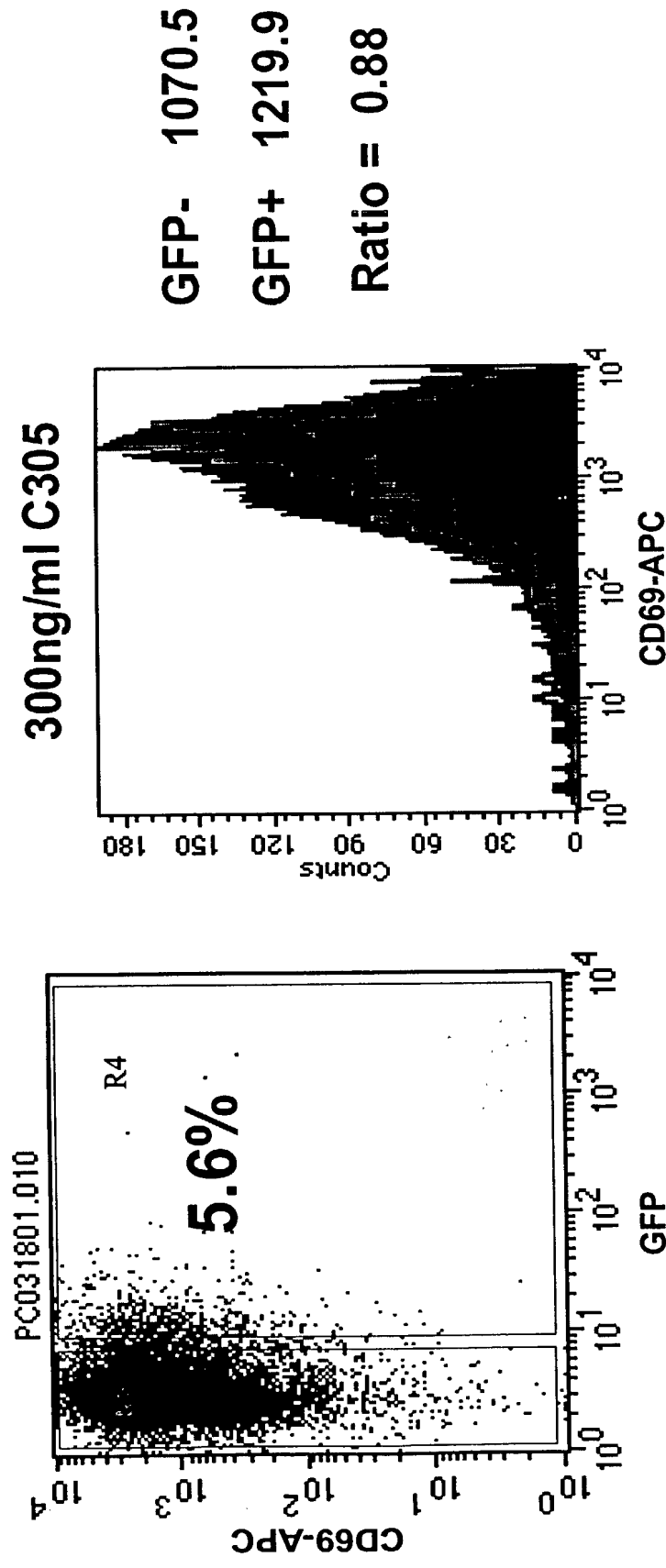


Figure 4A

FLJ20456 Hit Specifically Inhibited T Cell Activation but not B Cell Activation

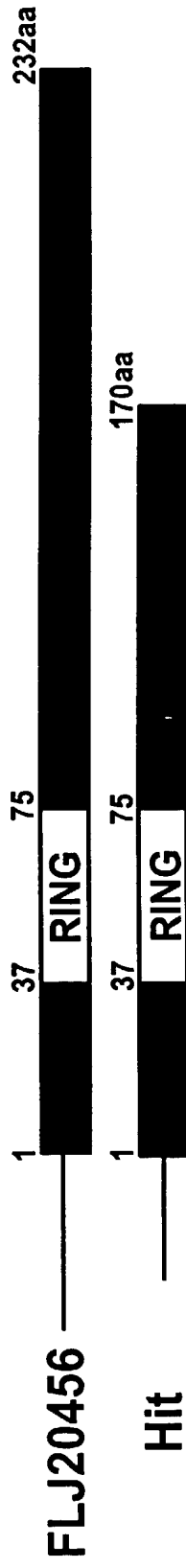


Figure 4B

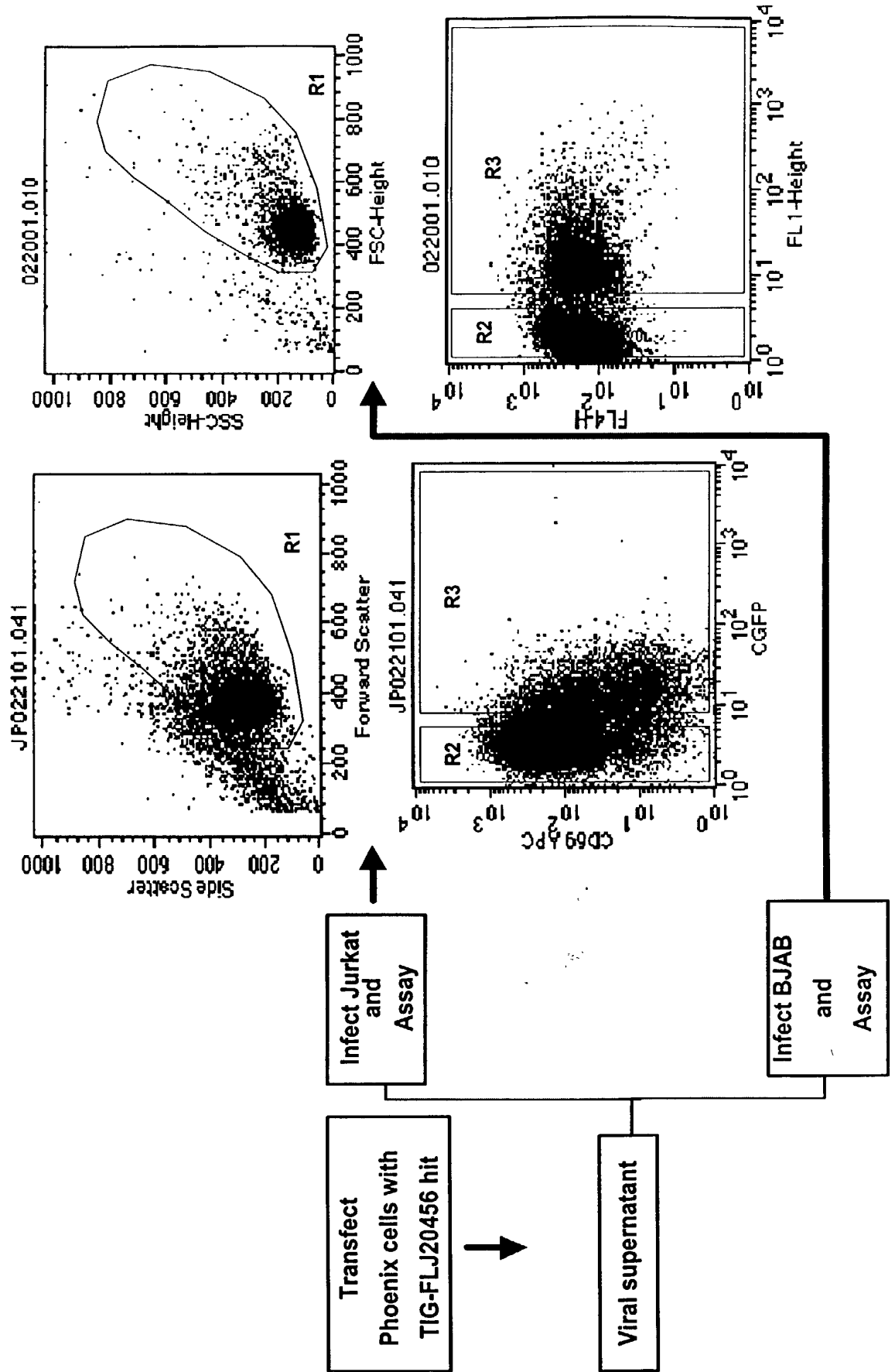
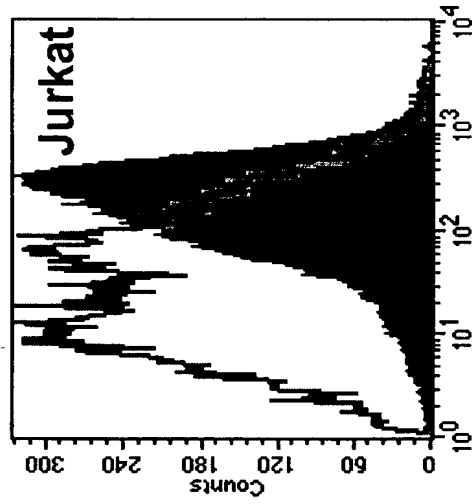


Figure 4C

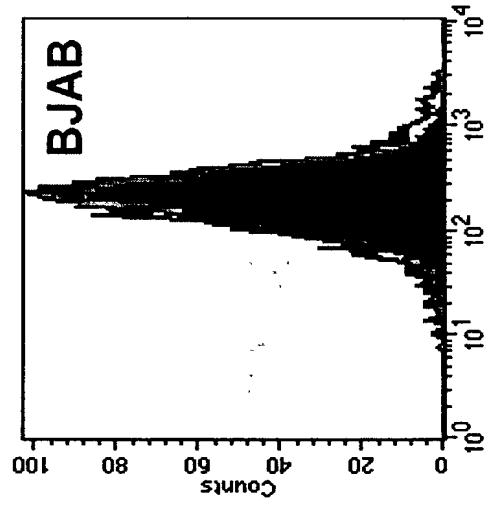


Stimulation:
300ng/ml C305

GFP- 141.2

GFP+ 28.8

Ratio = 4.9



Stimulation:
300ng/ml α-IgM

GFP-183.1

GFP+ 183.3

Ratio = 1.0

Figure 5

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs

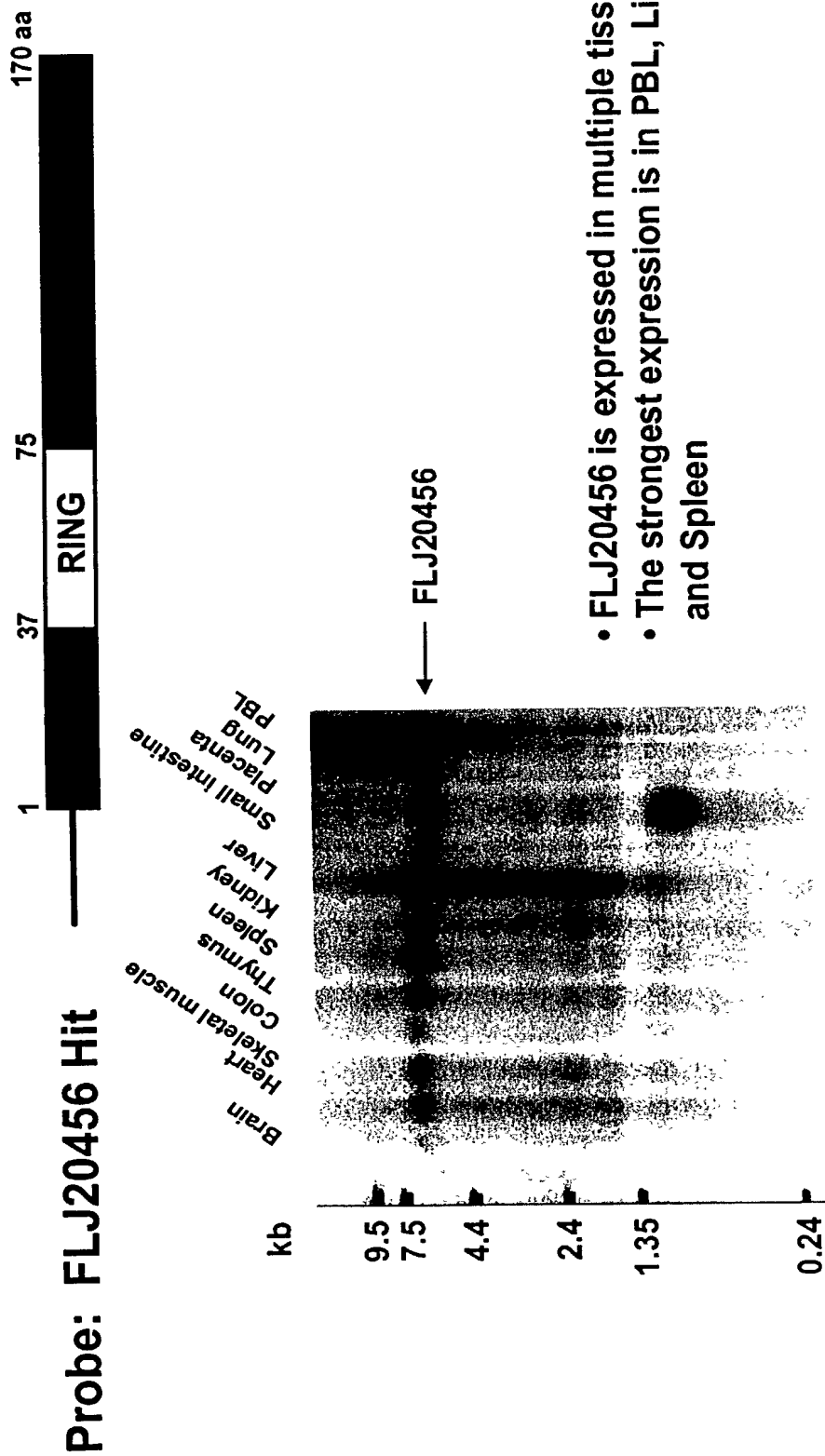
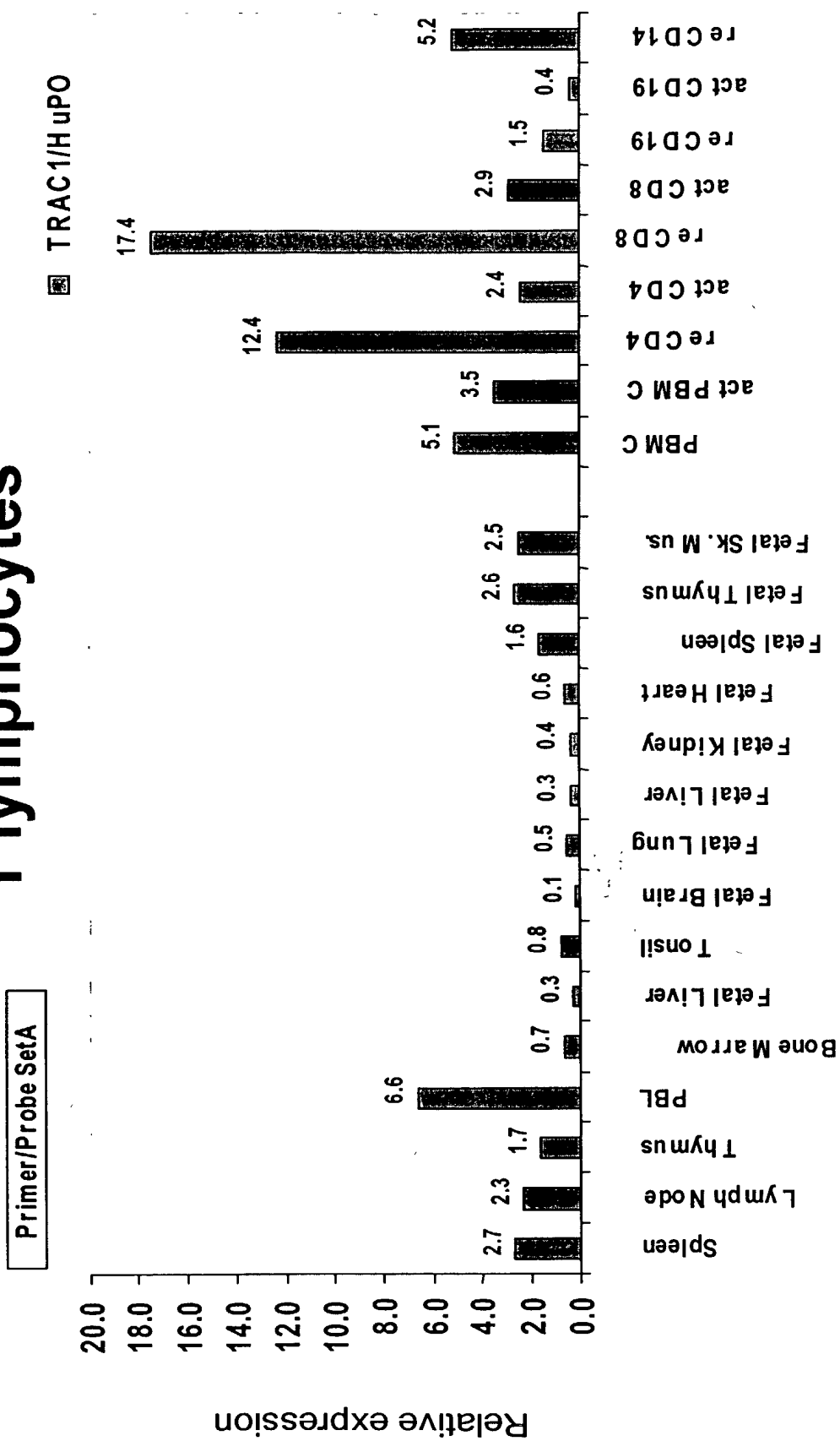


Figure 6

TRAC1 is Predominantly Expressed in Resting T lymphocytes



2006-2007 2008-2009 2009-2010 2010-2011 2011-2012 2012-2013 2013-2014 2014-2015 2015-2016 2016-2017 2017-2018 2018-2019 2019-2020 2020-2021 2021-2022 2022-2023 2023-2024 2024-2025 2025-2026 2026-2027 2027-2028 2028-2029 2029-2030 2030-2031 2031-2032 2032-2033 2033-2034 2034-2035 2035-2036 2036-2037 2037-2038 2038-2039 2039-2040 2040-2041 2041-2042 2042-2043 2043-2044 2044-2045 2045-2046 2046-2047 2047-2048 2048-2049 2049-2050 2050-2051 2051-2052 2052-2053 2053-2054 2054-2055 2055-2056 2056-2057 2057-2058 2058-2059 2059-2060 2060-2061 2061-2062 2062-2063 2063-2064 2064-2065 2065-2066 2066-2067 2067-2068 2068-2069 2069-2070 2070-2071 2071-2072 2072-2073 2073-2074 2074-2075 2075-2076 2076-2077 2077-2078 2078-2079 2079-2080 2080-2081 2081-2082 2082-2083 2083-2084 2084-2085 2085-2086 2086-2087 2087-2088 2088-2089 2089-2090 2090-2091 2091-2092 2092-2093 2093-2094 2094-2095 2095-2096 2096-2097 2097-2098 2098-2099 2099-2100 2100-2101 2101-2102 2102-2103 2103-2104 2104-2105 2105-2106 2106-2107 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3107-3108 3108-3109 3109-3110 3110-3111 3111-3112 3112-3113 3113-3114 3114-3115 3115-3116 3116-3117 3117-3118 3118-3119 3119-3120 3120-3121 3121-3122 3122-3123 3123-3124 3124-3125 3125-3126 3126-3127 3127-3128 3128-3129 3129

FL20456 Sequence is Most Similar to Two Sequences: Znf313 and STRIN

Ring Domain

Consensus #1F.C.VC.EV...PV....C.HVFC..C.....

FLJ20456.ppe MGSVLSTDSGKSAPASATAPATERRRRDPPELPTVSTFCVAVCLEVLHQPVPTTRCGHVFCRSCIAITSLKN 67

znf313.ppe -----MAAQQRDCGGAQIAGFAAEADPLGRFTCPVCLVEYKPVQV-PCGHVFCSAQLQECLKP 59

STRIN.ppe -----MAEDLSAATSYTEDEDEYCEVQDEVLKTPVPTTACQHVFCRKCFLTAMRE 49

Consensus #1C..CR.....A.....C..C.....R.H...C.KY...Y.....

FLJ20456.ppe NRTCPYCRAYTPSE---GVPTDVAKPMKSEYKNCAECDTLVCLSEMTAHIRTQCKYIDKYGPLQE 131

znf313.ppe KKPVCGVCRSALAP-----GVRVLELEROIESTETSGHGRNFFLSKIRSHVATCSKYON-YIMEGV 121

STRIN.ppe SGACHPLCRGNVTRRERACPERALDENIMRKFSGRCRCAKQIKFYMRTHHYKSCKKYQDEYGVSSI 117

Consensus #1CP.C.....L..HC...H.....

FLJ20456.ppe LEETAAR-----CVCPCQRELYE-DSLDDHCITHTHSERR 166

znf313.ppe KATIKDASLQPNVPNRY-----TFPCPYCEPKNFDDQGLVEHCKLHFSTDTK 169

STRIN.ppe VPNFQISQDSVGNRSETSTSDNTETTYQENTSSSGHPTFKCPLCQESNFTRQRLDDHCNSNHLFQIV 185

Consensus #1 .V.CP.C...P...P....N...H.....Y..F.....EE.....S.....-

FLJ20456.ppe PVFCPLCRLIPDENPSSFSGNLIRHLQVSHTLFYDDFLDFNIEEALIRRVLDRLSLLLEVNHSTNT. 233

znf313.ppe SVMCPICASMPWGDPNYRSANFRHTQRRHRESYDTFVDYDVDEEDMMNQVLORSIIDQ. 229

STRIN.ppe PVTCPICVSLPWWGDEPQITRNFVSHLNORRQEDYGEEMNLQIDDEETQYQTAVEESFQVNI. 246

* All three sequences are human
 * Murine sequences are not shown

| | 1 | 2 | 3 | |
|---|-------|-------|------|--------------|
| 1 | | 26.6 | 22.3 | 1 |
| 2 | 130.4 | | 27.9 | 2 |
| 3 | 140.9 | 134.7 | | 3 |
| | 1 | 2 | 3 | |
| | | | | FLJ20456.ppe |
| | | | | znf313.ppe |
| | | | | STRIN.ppe |

FIG. 7.

Alignment of RING Domain Sequences from Various Human Proteins

| Consensus #1 |C..C.....C.H..C..C.....C.....C..... |
|---------------|---|
| FLJ20456.Ring | VTSFDCAVCLEVLHQVPVTR-CGHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |
| znf313.Ring | LGRFTCPVCLVEYEKPVQVP-CGHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |
| STRIN.Ring | EDDFYCPVCEVLKTPVTTACQHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |
| TRAF6.Ring | ESKYECPICLMALREAVQTP-CGHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |
| c-Cbl.Ring | STFQLCKICAEEN-DKDVKIEPCGHLMTSCLTSW--QESEGGQCPFCRCEI-KG 50 |
| BRCA1.Ring | ---LECPICLELIKESVTK-CGHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |
| BAR.Ring | VSEFSCHCCYDILVNPT-TLNCGHSFCHCLALWWA--SSKKTECPCECREK--WE 50 |
| RAG1.Ring | VKSISQICEHILADPVEIN-CGHVFCRSCIAATSL--KNNKWTCEPYCRAYLP-S 50 |

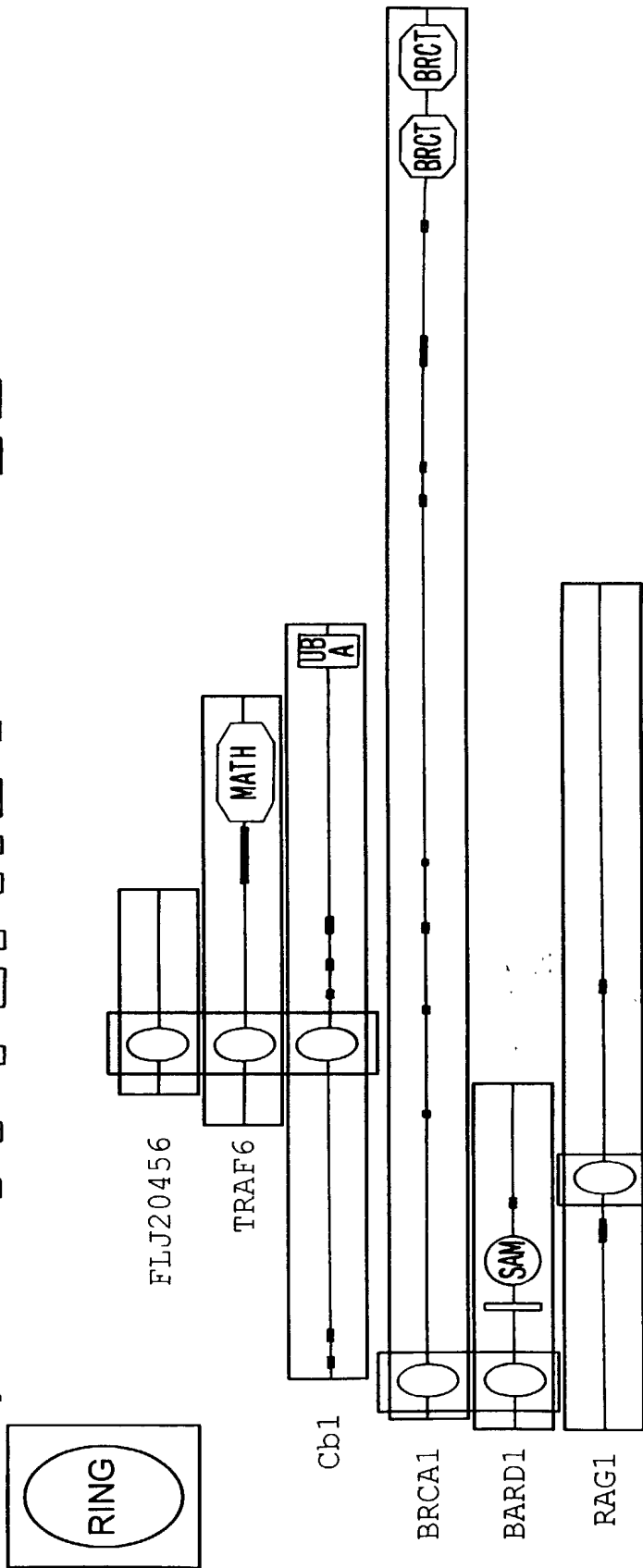


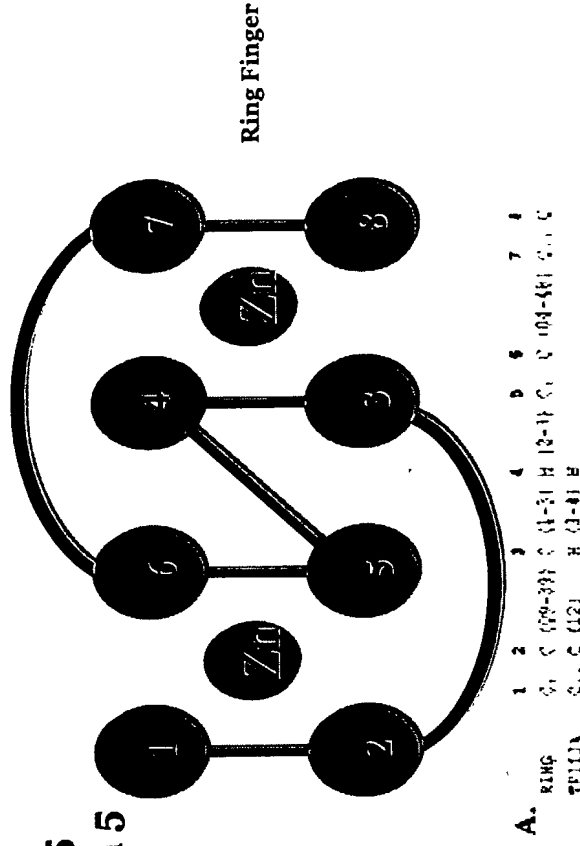
FIG. 8.

RING finger vs. Zinc finger proteins

Figure 9

Ring-HC: C_3HC_4 = Cys in position 5
 Ring H2: $C_3H_2C_3$ = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that coordinate two zinc atoms to form a cross-brace structure



- Ring fingers are structurally distinct from zinc fingers

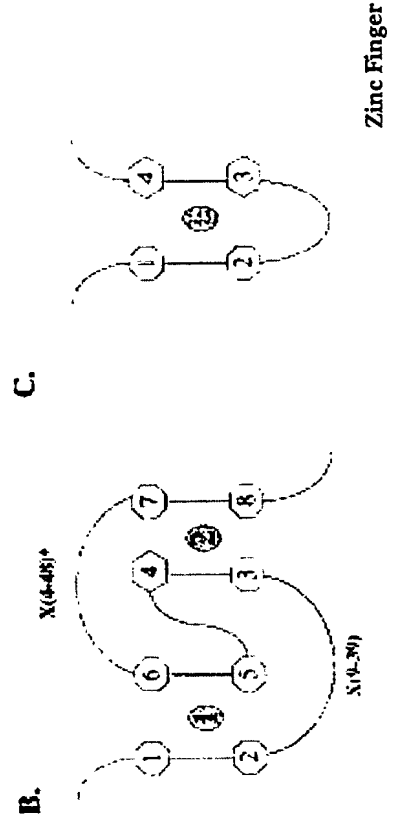


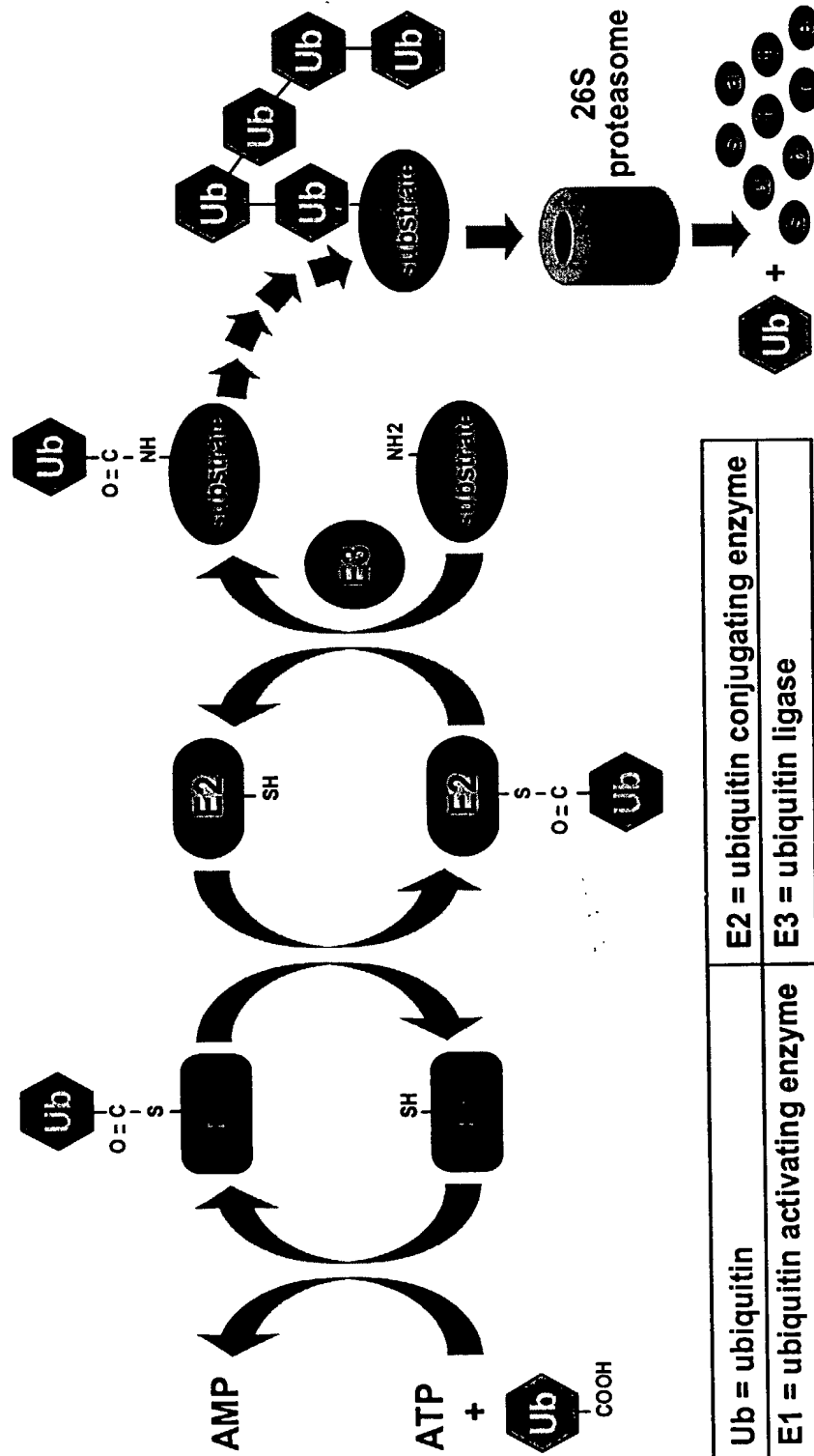
Figure 10A

Ubiquitin Pathway Components

- E1: ubiquitin-activating enzyme, with a major isoform that may work broadly
- E2: ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3
- E3: ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins
- Proteasome-a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.

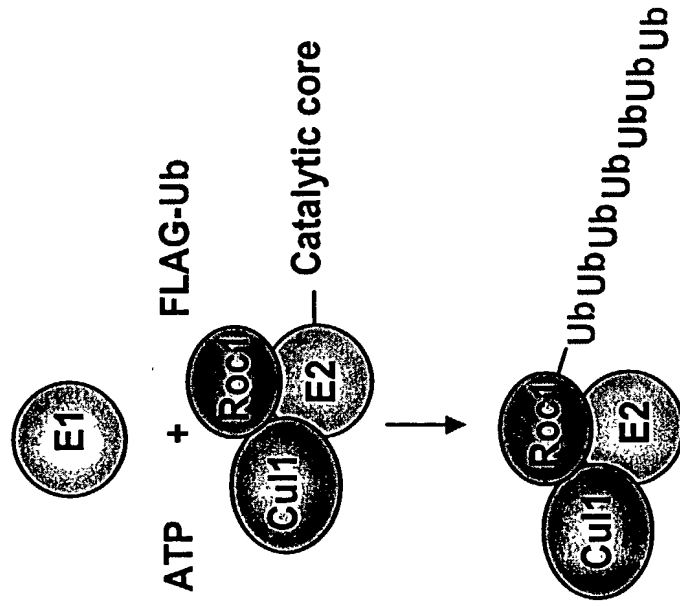
Figure 10B

Enzymology of Ubiquitination



A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis

Figure 11A



The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Reaction Components

13

E2 (Ubch5): GST-fusion (cleaved), E.coli

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus

Ubiquitin: FLAG-tagged, E.coli

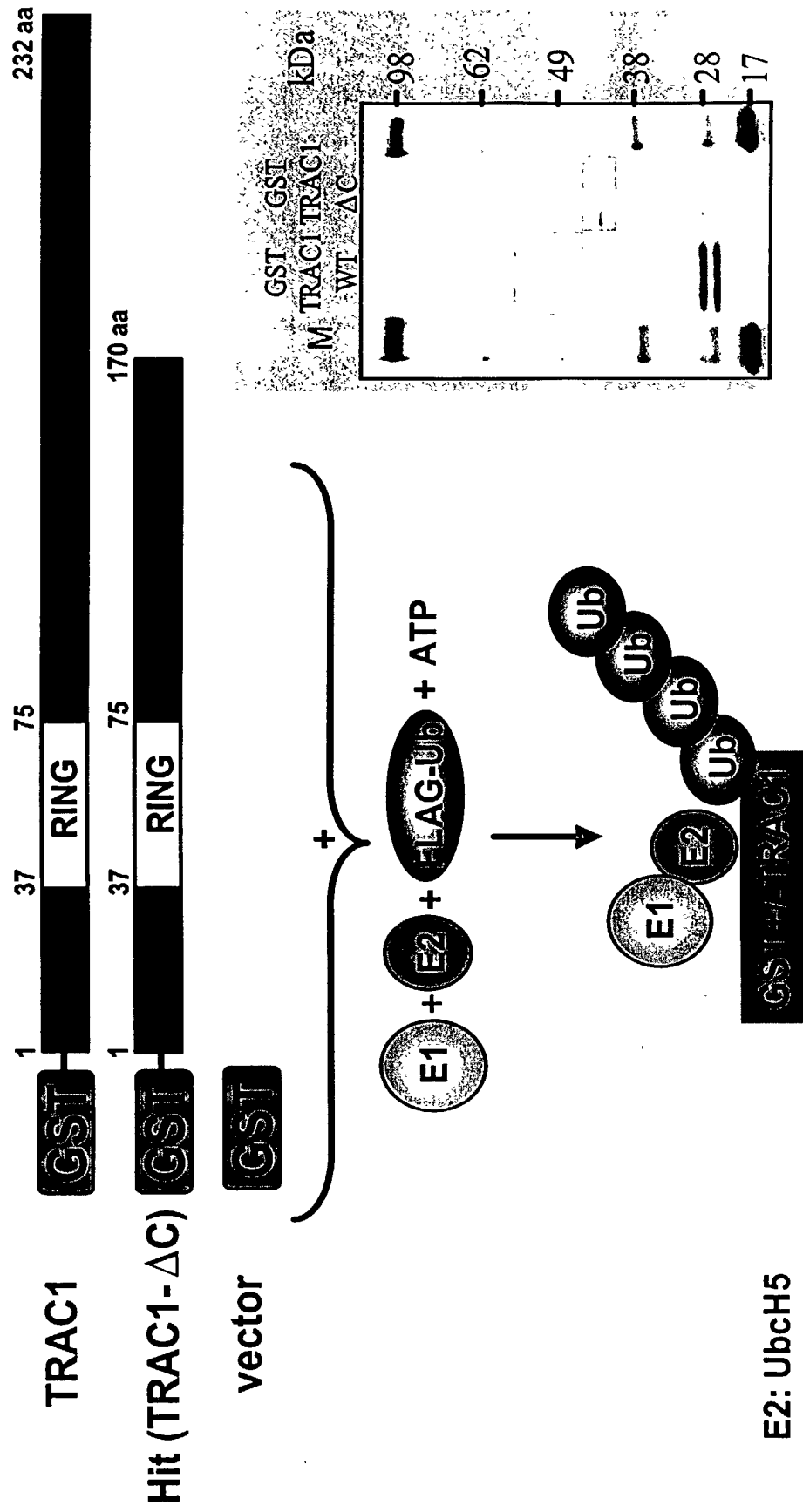
Reaction lacking:

E1 E2 E3 Ub ATP

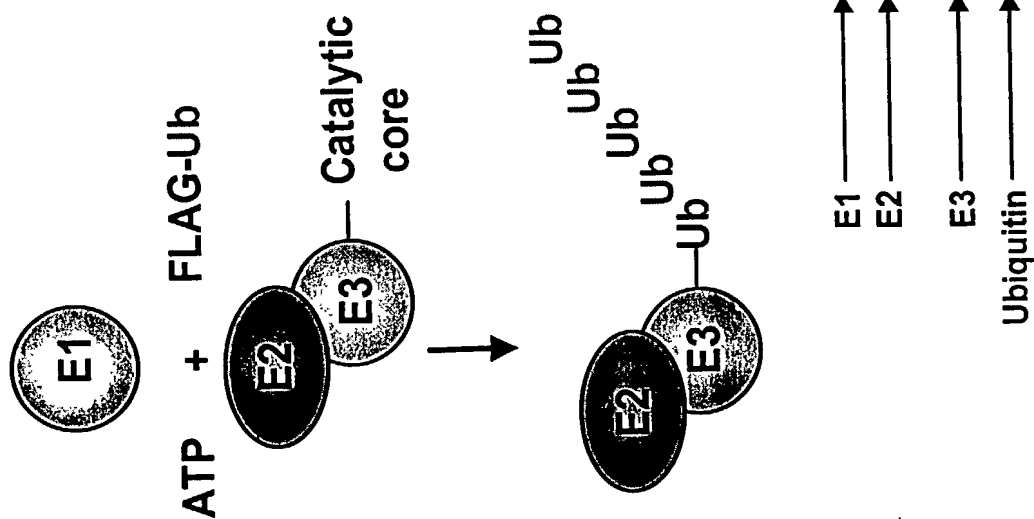
FLAG-Ub_N

FLAG-Ub

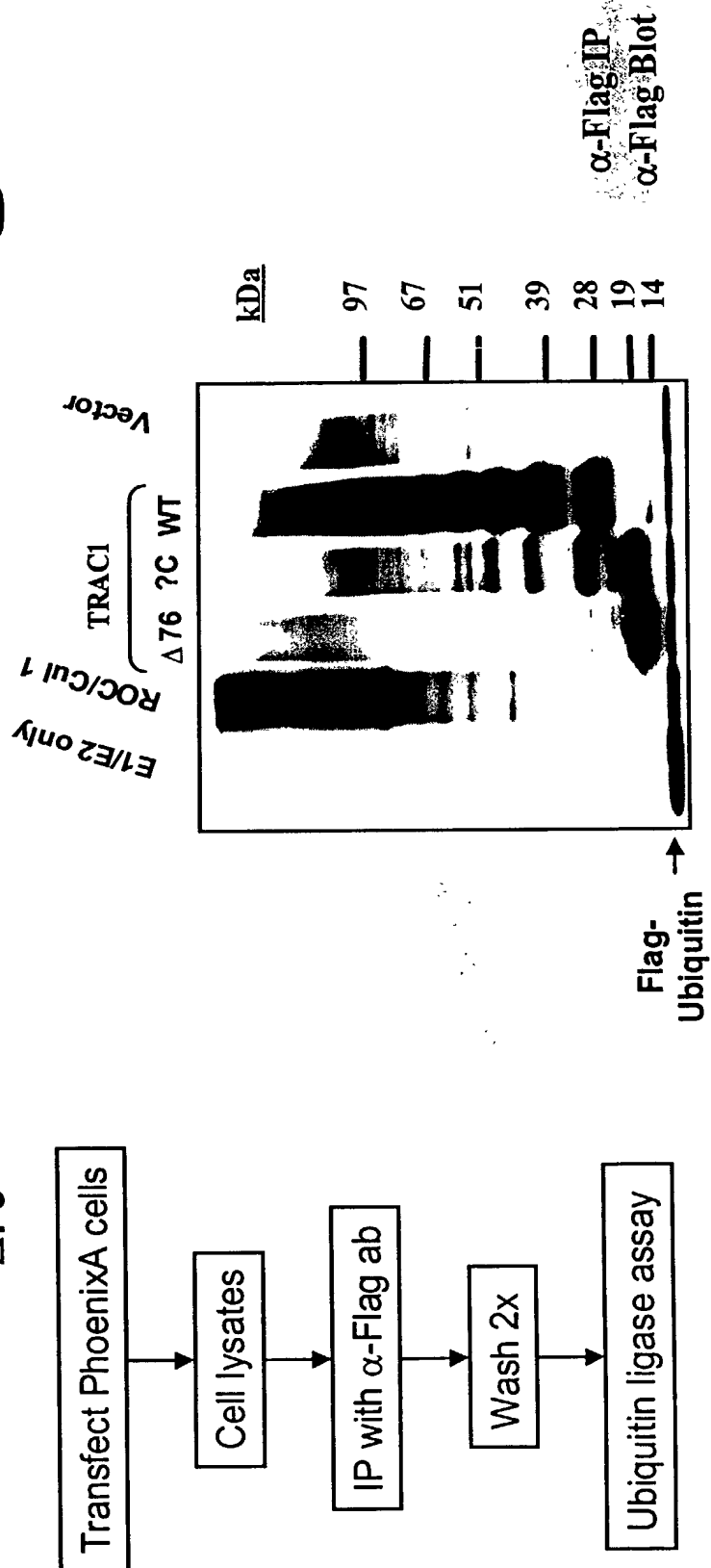
Figure 11B Bacterially-expressed TRAC1 for Ligase Activity Assay



(b) The following information shall be furnished by the contractor:

[illegible]

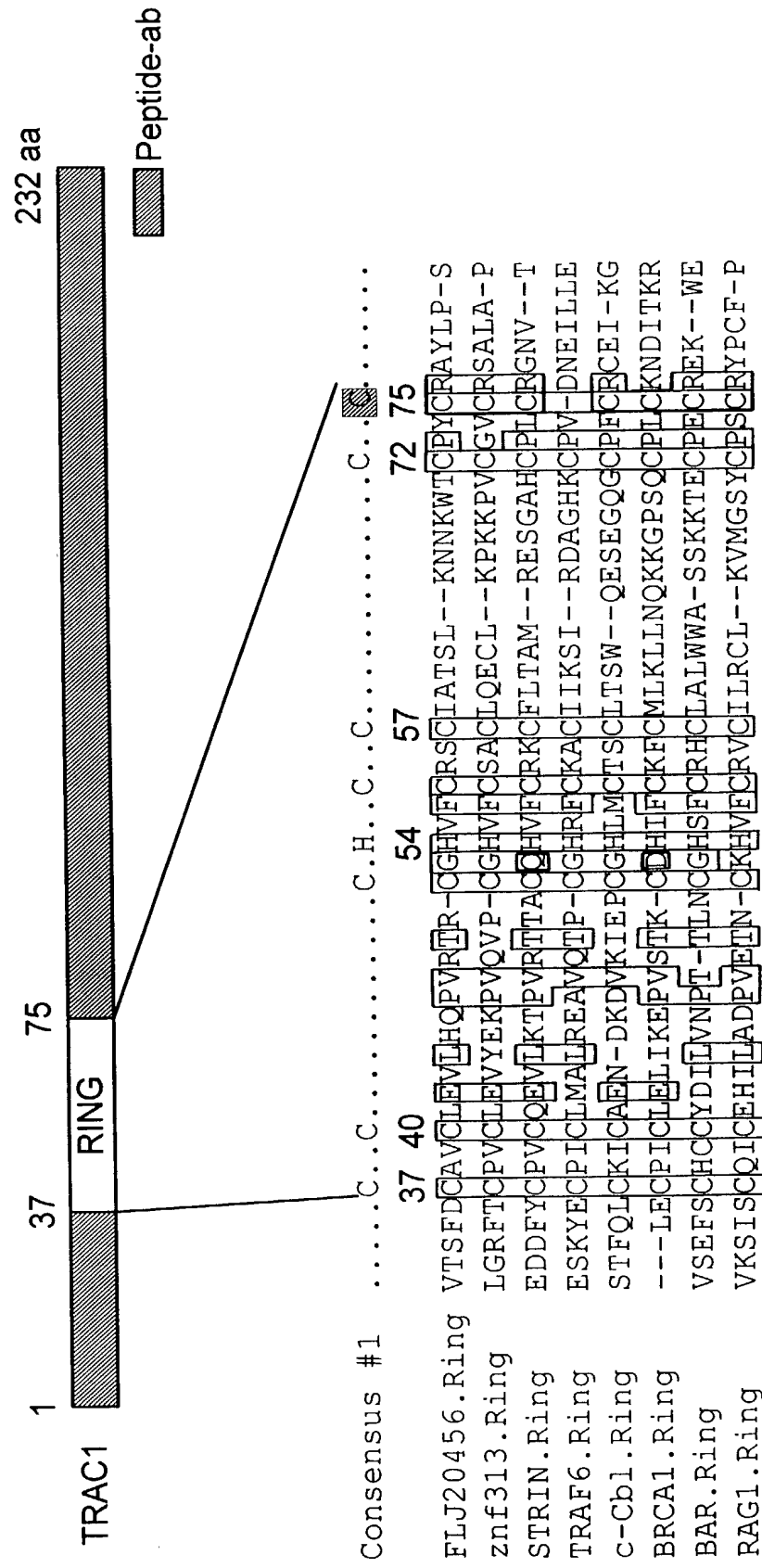
The Ring Domain is Required for TRAC1 Ligase Activity



Flag-Ubiquitin

+

Point mutations in Conserved Cysteine Residues of the TRAC1 Ring finger Domain



- The following expression plasmids were generated:
pEFnig/Ring finger point mutants: H54A, C75A, C37, 40A, H54C57A, C72, 75A
pEFnig/Myristylation site mutant: G2A

FIG. 13A.

+

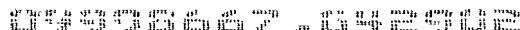
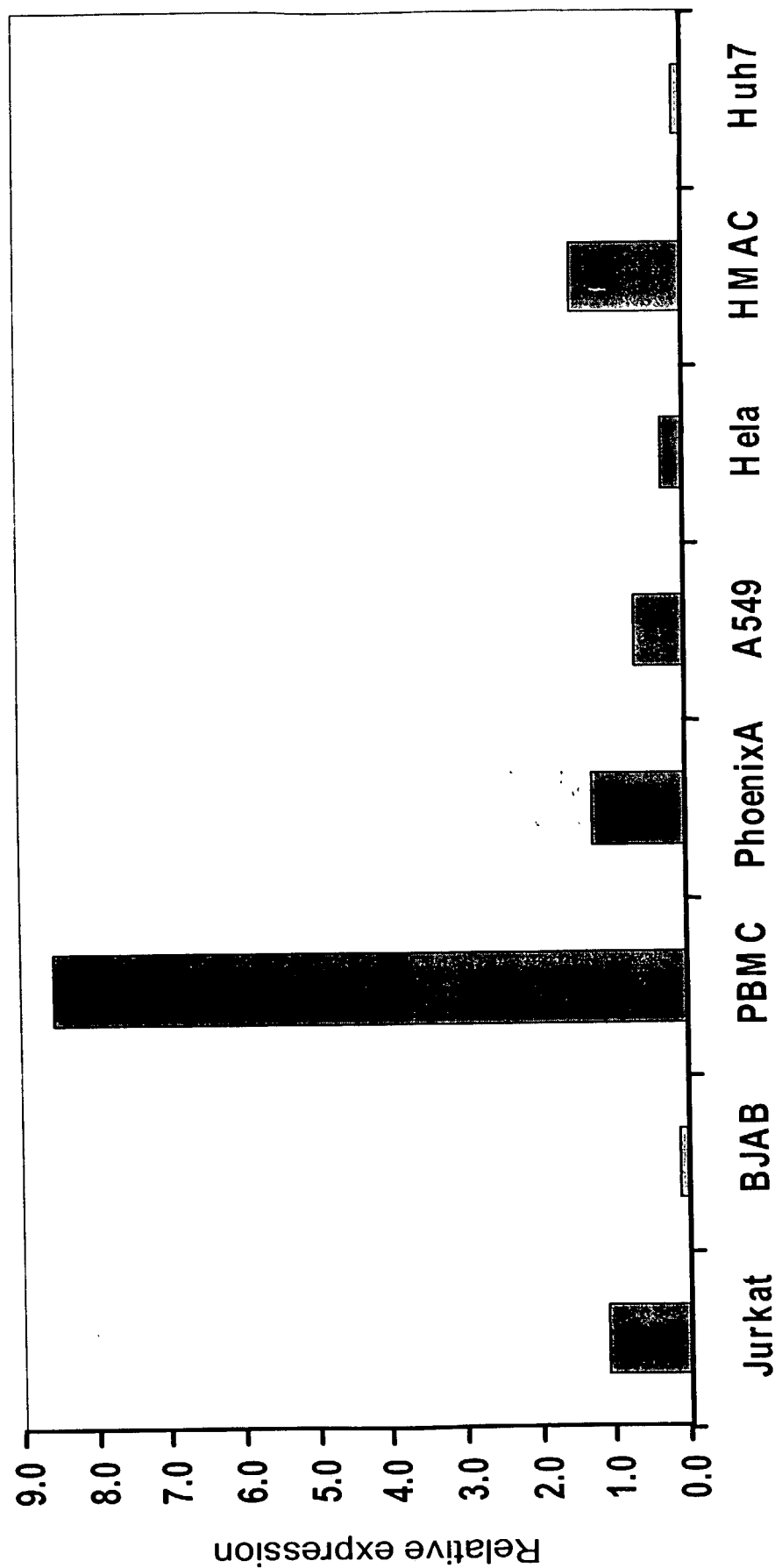
[illegible][illegible]

Figure 14

**Expression of TRAC-1 mRNA is ~8 fold
higher in PBMC than in Jurkat cells**

■ TRAC1/HuPO



0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0

Figure 15A

C-terminal Truncated TRAC1 Blocks TCR-induced Ca^{2+} Influx

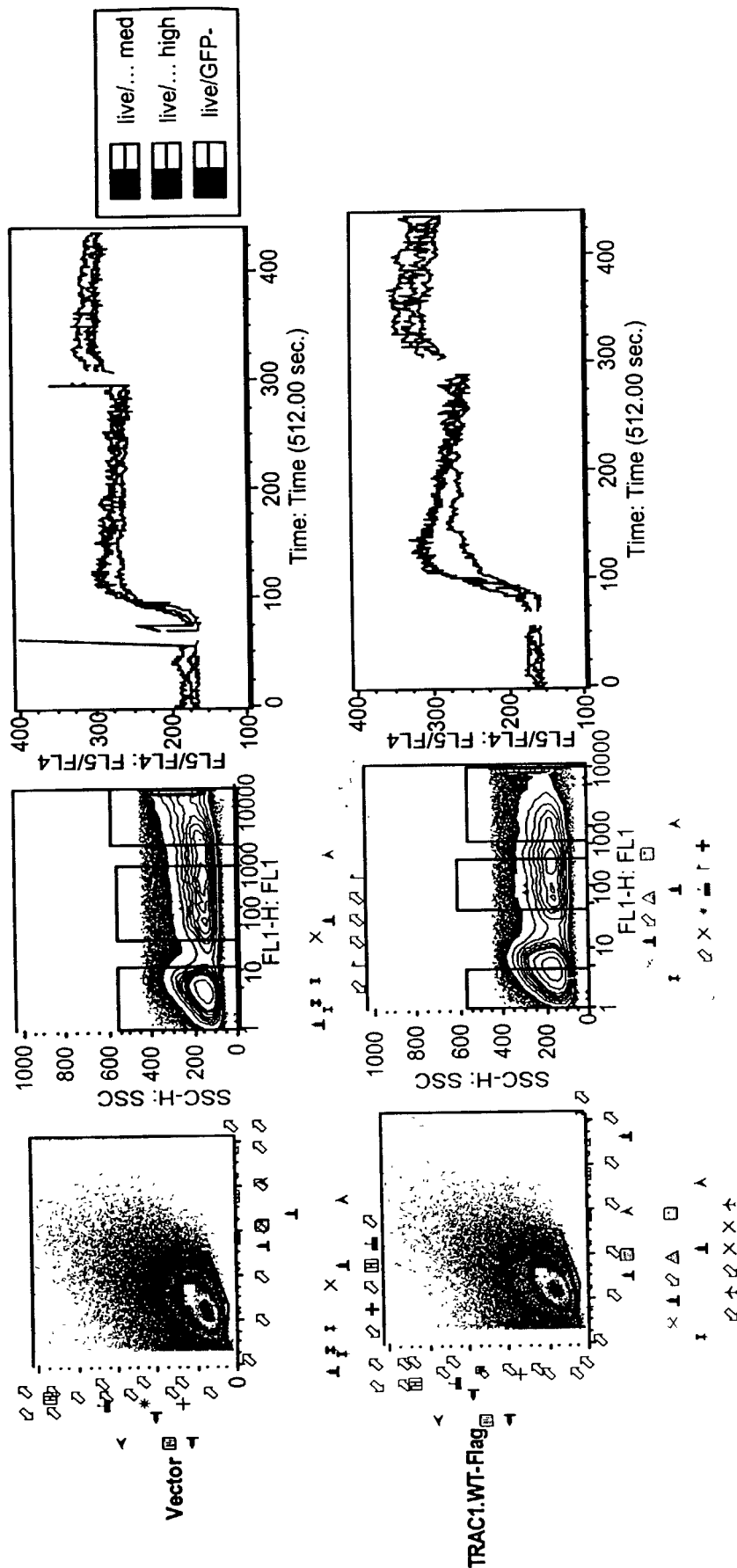


Figure 15B

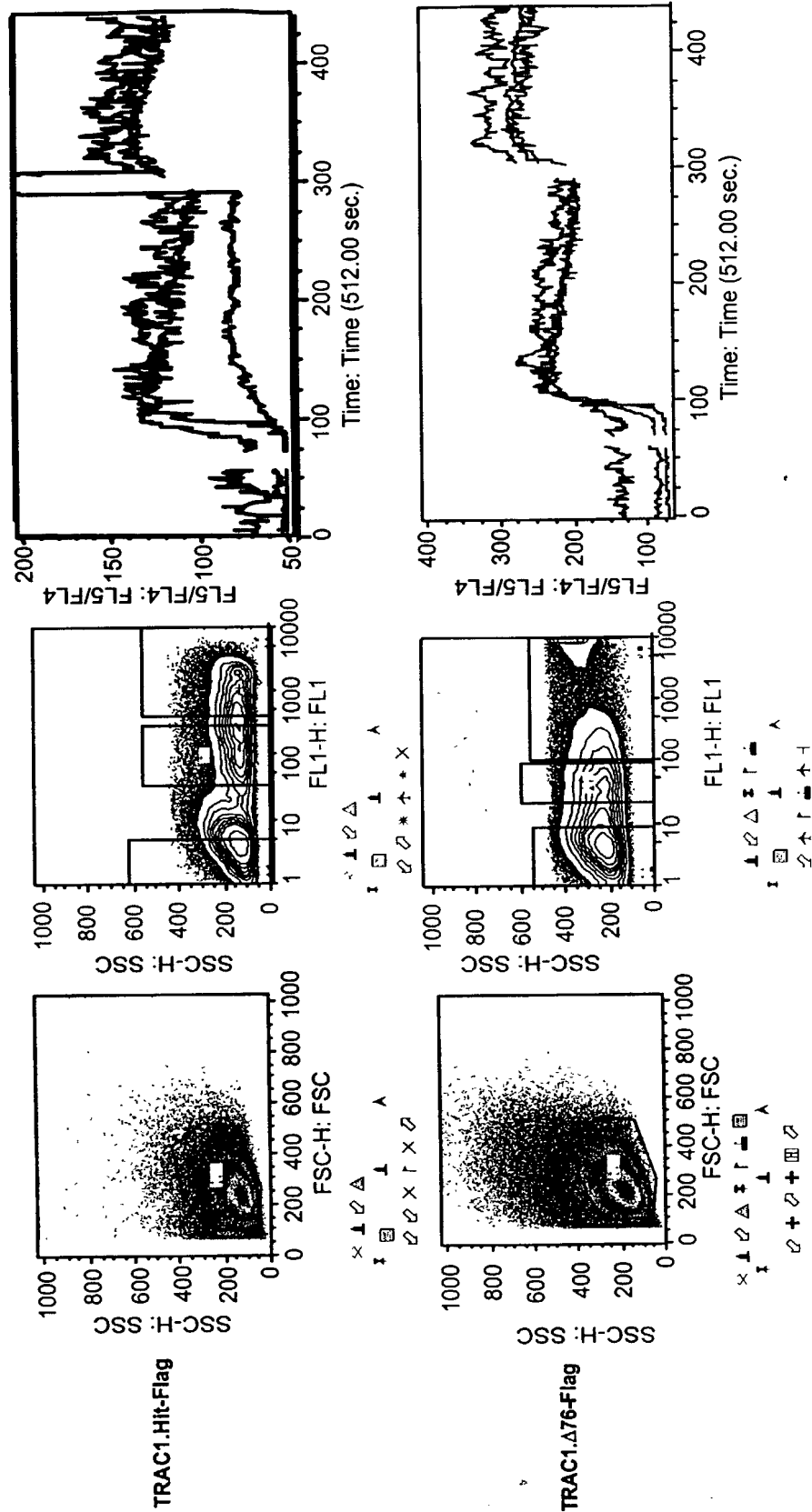


Figure 16

An Intact TRAC1 Ring domain is Required for Inhibition of α -TCR-Induced CD69 Up-regulation

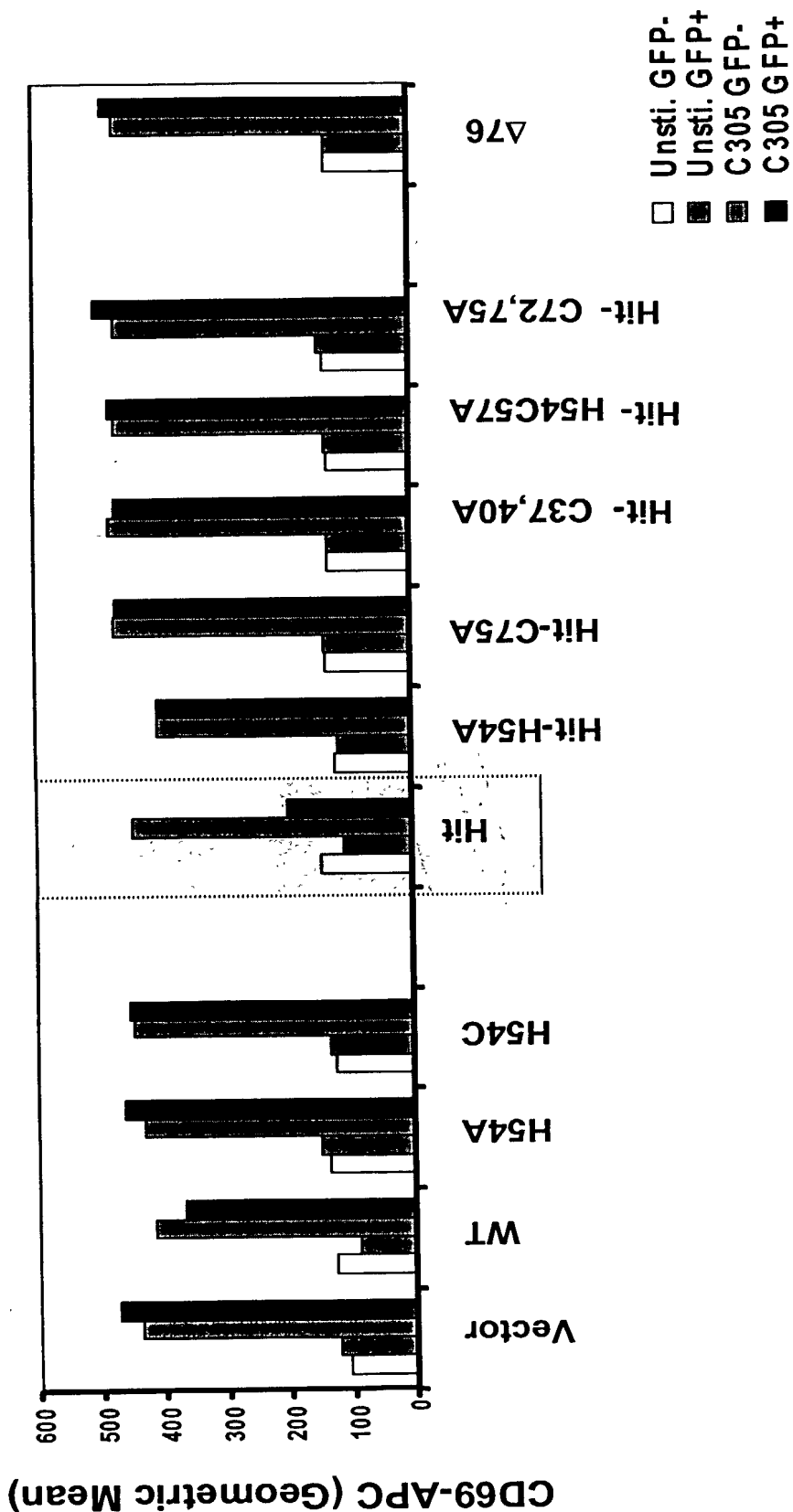


Figure 17

Summary of Functional Effects by Different TRAC-1 constructs

| | | Ubiquitin ligase activity | CD69 induction | Calcium mobilization |
|---------|-----------------------------|---------------------------------|-------------------|-------------------------|
| TRAC1 | 1 37 75 232 aa RING Flag | yes | - | +/- |
| Hit | 1 37 75 170 aa RING Flag | yes | → | → |
| Δ76 | 77 232 aa Flag | no | - | - |
| C37,40A | X RING Flag | | - | - |
| C72,75A | RINX Flag | | - | - |
| H54C57A | RXXG Flag | | - | - |

33333333 2 1042933

Figure 18

Transiently Transfected TRAC1 Protein Binds to Ubiquitin - Conjugating Enzymes (E2s) UbcH7 and UbcH5 *in vitro*

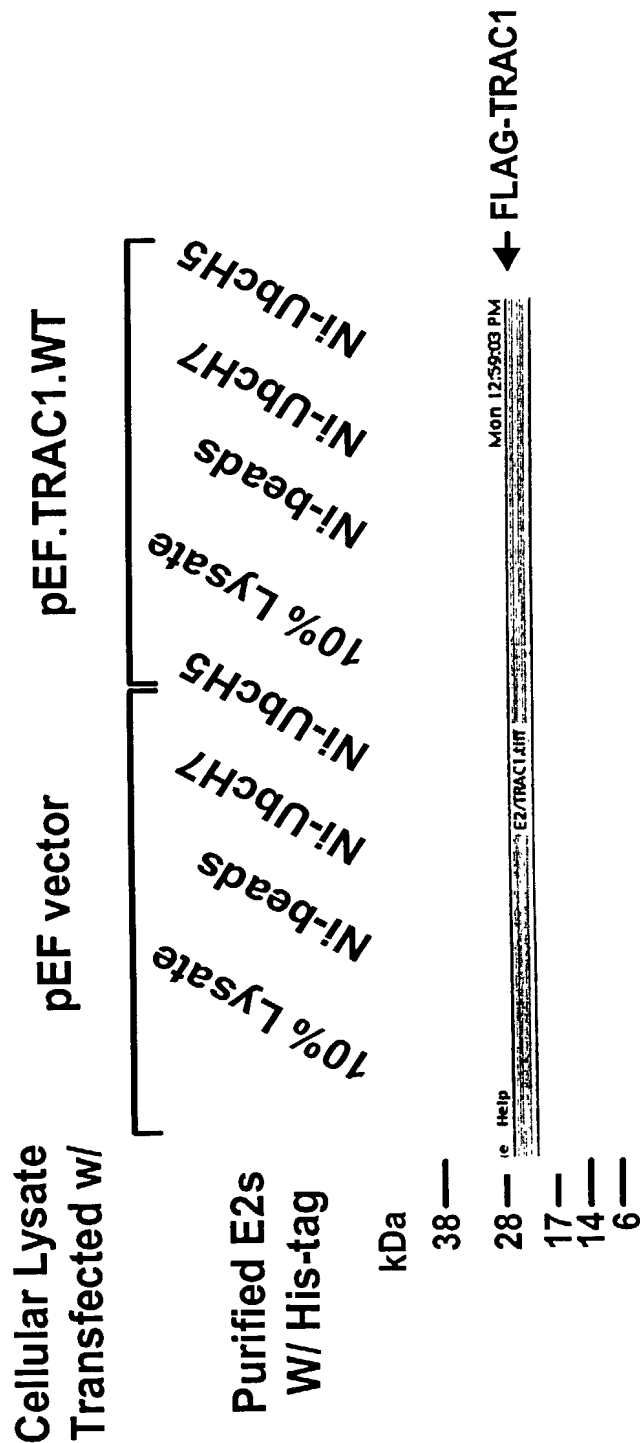
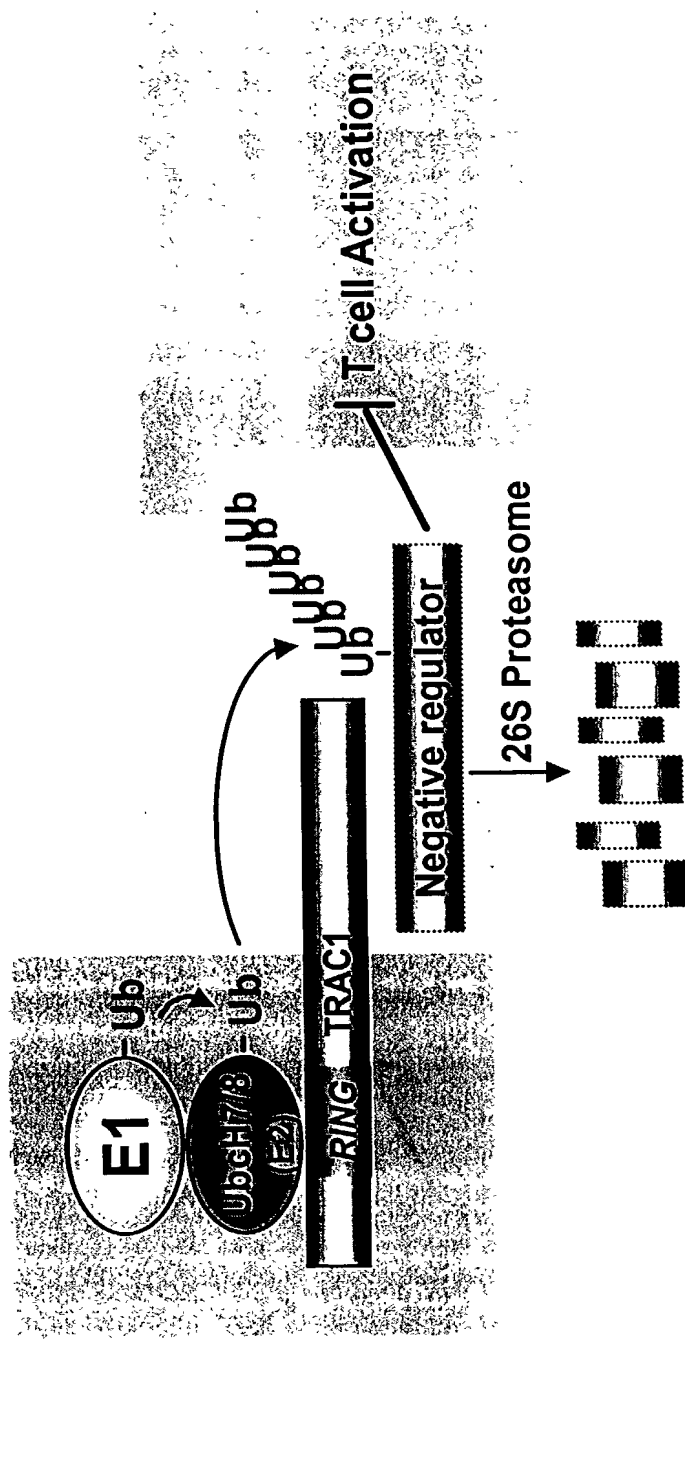


Figure 19

Model for TRAC-1 regulation of T cell activation



TRAC1